Inhibition of cell migration and endothelial cell tubulogenesis by compounds found in black raspberries

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for Graduation *with research distinction* in Biology in the Undergraduate Colleges of The Ohio State University

By

Colleen M. McIntyre

The Ohio State University

August 2007

Project Advisors: Dr. John Lechner and Dr. Gary Stoner,

Department of Internal Medicine

Abstract

Cell migration and endothelial cell tubulogenesis are both processes which are altered in many cancers. Black raspberries and their components have been shown to inhibit tumor growth and neovascularization *in vivo*, but the active components in black raspberries are still being identified. This experiment used two assays to determine the effect of black raspberry components on tumor cell migration, endothelial cell migration, and endothelial cell tubulogenesis. Ellagic acid and quercetin effectively inhibited migration of both cell types. Human Umbilical Vascular Endothelial Cell (HUVEC) migration was inhibited by ellagic acid at 2 μM (30.9% control ± 10.1%, $P \leq 0.04$), and by quercetin at 10 μM (24.1% control ± 2.5%, $P \leq 0.02$). Migration of the esophageal tumor cell line (TE-8) was studied to evaluate differences between endothelial cell and tumor cell sensitivity to each compound. Migration of TE-8 cells was inhibited by ellagic acid at 2 μM (43.1% control ± 15.9%, $P \leq 0.04$), and by quercetin at 20 μM (40.3% control ± 18.7%, $P \leq 0.05$). Both ellagic acid and quercetin inhibited endothelial cell tubulogenesis qualitatively at 2 μM. However, HUVEC morphology differed between the two treatments. Ellagic acid inhibited tubulogenesis and cell elongation at 2 μM, whereas quercetin inhibited tubulogenesis, but not cell elongation at 2 μM.

Acknowledgements

I would like to thank my project advisors Dr. John Lechner and Dr. Gary Stoner for their limitless help, patience, and resourcefulness. Special thanks to Nancy Zikri, Li-Shu Wang, and all the other researchers at The Ohio State University Polaris Innovation Center; it has been a pleasure to work with you.

Introduction

Many foods are known to possess anti-carcinogenic properties and can be used as nutraceutical foods for cancer prevention, or developed into chemotherapeutic agents. Like many berries, the black raspberry (*Rubus occidentalis*, BRB) contains both known and suspected anti-carcinogenic compounds, such as anthocyanins, ellagitannins, flavanoids, and flavonoids[1,2,3]. Current research indicates that the anti-carcinogenic activity observed in black raspberries may be a synergistic effect of more than one compound acting on multiple cellular pathways, because direct extracts have more activity than most fractions or isolated compounds alone[4,5]. However, specific compounds with anti-carcinogenic activity in black raspberries have been identified, such as cyanidin glycosides, ellagic acid, gallic acid, and quercetin[6,7,8]. At least three cyanidin glycosides, a type of anthocyanin, are known to inhibit nuclear factor kappa B (NFκB) and activator protein-1 (AP-1), which play a role in carcinogenesis[9].

This study examined the anti-migratory and anti-angiogenic effects of a cyanidin chloride derivative, keracyanin chloride. Studies have shown that ellagic acid, a phenolic compound, inhibits endothelial cell migration and tubulogenesis, but little is known about

the synergistic effect it may have in BRBs[10]. The effects of ellagic acid were compared to two BRB fractions in order to determine if a synergistic effect was taking place. The flavonoid quercetin also has known anti-carcinogenic effects, such as tyrosine kinase inhibition, matrix metalloproteinase-2 inhibition, and free-radical scavenging[11]. The purpose of this study was to compare the anti-migratory and anti-angiogenic capability of pure ellagic acid, keracyanin, and quercetin to two BRB fractions in order to determine if a synergistic interaction was taking place, and to examine differences between each treatment that could indicate different cellular mechanisms were being affected.

Although cancers vary greatly in genotype, there are commonalities in the cellular processes that become altered during transformation of a normal cell into a malignant cancer. Two of these processes are angiogenesis and cell migration; both processes are limited in normal cells, and are commonly acquired during carcinogenesis. Because oxygen and nutrient diffusion to cells within the body is limited by proximity to the supplying vascular tissue, tumors larger than 1-2 mm^3 must acquire the ability to induce angiogenesis in order to continue growth[12]. Angiogenesis in human adults is normally limited, and occurs only during events such as menses and wound healing[13]. This makes prevention of tumor neovascularization a good potential target for chemopreventative agents. Anti-angiogenic treatment is also attractive because an endothelial cell's genome is more stable than that of a tumor cell, so drug resistance is less likely to develop[14]. The most effective treatments will most likely combine anti-angiogenic treatment with more traditional chemotherapy aimed at prevention of tumor metastasis. Tumor metastasis depends on the ability of the cancer cells to migrate to other locations in the body, and is generally associated with poor prognosis[15]. This is a complicated process, and may be further complicated by individual differences between tumors. The process of metastasis starts when tumor cells detach from the primary tumor. This may be due to a number of cellular factors, or necrosis in the primary cell mass[16]. Tumor cell detachment is followed by migration into the surrounding tissue (intravasation), and migration into vascular tissue (extravasation). Most of the tumor cells will die, but those that survive may circulate to a new location in the body, extravasate again, and form a new tumor[17]. Chemotherapeutic agents that inhibit cell migration may be able to prevent metastasis, and limit the scope of the cancer.

The purpose of this project was to test potential chemotherapeutic agents in the components of BRBs for the *in vitro* inhibition of cell migration in human esophageal tumor cells (TE-8 cells), and the inhibition of either cell migration or tubulogenesis (microvessel formation) in human umbilical vascular endothelial cells (HUVECs).

Materials & Methods

Compounds. Anthocyanin (keracyanin chloride), ellagic acid, and quercetin (dihydrate) were obtained as pure compounds (Sigma, St Louis, MO). Two black raspberry fractions were obtained from a previous experiment (courtesy of Dr. Gary Stoner)[18]. The 80/20 fraction was extracted from lyophilized BRBs using 80% ethanol and 20% water. The

residue fraction consisted of the lyophilized BRB remainder after extractions with pentane, methylene chloride, ethanol, and water. Each compound was initially dissolved in filtered dimethylsulfoxide (DMSO), then diluted in Medium 131 (Cascade Biologics, Portaland, OR) to the appropriate concentration. The concentration of DMSO was kept below 0.1% (v/v).

Cell Migration Assay. TE-8 cells (a kind gift from Xiao-Chun Xu)[19] and HUVECs (Cascade Biologics, Portland, OR) were each seeded into 24 well culture plates and allowed to grow to confluence. Two perpendicular lines were scraped through each well with a 1mm wide cell scraper (Fisher Scientific, Pittsburgh, PA). The cells were rinsed 2x with Hepes Buffered Saline (Athena ES, Baltimore, MD) before adding anthocyanin, ellagic acid, 80/20 fraction, quercetin, or residue fraction at 0, 1, 2, 5, 10, 20, and 30 μM (μg/ml was used for the fractions). Each concentration was assayed in duplicate. The cells were incubated 48h at 37°C, fixed for 10 min in buffered formalin, and stained with 0.3% crystal violet for 5 min. Computer image analysis (Simple PCI, Compix Inc., Sewickley, PA) was used to count the total number of cells in representative views of each well (4 images per concentration, taken at 40x), and to enumerate the number of cells in a 1x1mm area which had been scraped (fig. 1). The standard error of the mean was determined for each point, and the P value determined using a two-tailed t-test assuming equal variances.

Cytotoxicity Assay. Ellagic acid and quercetin were tested for cytotoxicity in both cell types using the CCK colorimetric assay (Dojindo Molecular Technologies, Gaithersburg, MD) in a 96 well plate. Cells were seeded at 5,000 cells/well, allowed to grow to confluence, and treated identically to the cell migration assay until completion of the incubation period. CCK-8 solution (10 μl/well) was added, and the plates were incubated at 37°C for 1 hour. The absorbance was measured at 450 nm. Absorbance was plotted relative to the control, standard error of the mean was determined for each point, and the P values were determined using a t-test, as above.

Tubulogenesis Assay. Matrigel (BD Biosciences, San Jose, CA) was diluted 1:1 with Medium 131on ice. Each compound to be tested was included at the appropriate concentration in the Matrigel layer. The diluted Matrigel was put into a 96 well culture plate (60 μl per well), and incubated 15 min at 37°C to allow gelling. Dissociated HUVECs were diluted in Medium 131 such that the cell concentration was 1 x 10^4 at each compound concentration. The compound/HUVEC mixture was added onto the Matrigel layer in each well(100 μl, or 1000 cells per well). Ellagic acid and quercetin were added at 0, 2, 5, 20, 50, and 100 μM in triplicate, and incubated at 37°C for 24h. The cells were fixed in buffered formalin for 10 min. Images were taken of the unstained cells(fig 2), and observed for cell elongation or tube formation qualitatively (quantitative data was not practical using current software).

Results

Cell Migration. Migration of both HUVECs and TE-8 cells was inhibited by treatment with ellagic acid and quercetin. Both cell types were affected by ellagic more than by treatment with quercetin. Anthocyanin, 80/20 fraction, and residue fraction showed no

statistically significant inhibition of either cell type at the concentrations used. HUVEC migration (fig. 3) was inhibited by ellagic acid at 2 μM (30.9% control ± 10.1%, $P \leq 0.04$), and by quercetin at 10 μM (24.1% control ± 2.5%, $P \leq 0.02$). TE-8 cell migration (fig. 3) was inhibited by ellagic acid at 2 μM (43.1% control ± 15.9%, $P \leq 0.04$), and by quercetin at 20 μM (40.3% control ± 18.7%, $P \leq 0.05$). HUVECs were more sensitive to quercetin than TE-8 cells at the same concentration.

Cytotoxicity. Quercetin was cytoxic in both cell types. Ellagic acid was cytoxic in HUVECs, but was not significantly cytoxic in TE-8 cells (fig. 4). Ellagic acid showed cytotoxicity in HUVECs at 1 μM ($P \leq 0.008$), but was not cytoxic in TE-8 cells at the concentrations tested. Quercetin was cytoxic in HUVECs at 5 μM ($P \leq 0.002$), and was cytoxic in TE-8 cells at 10 μM ($P \leq 0.02$).

Tubulogenesis. HUVEC tubulogenesis was qualitatively inhibited by both ellagic acid and quercetin at 2 μM. Cell elongation was observed in wells treated with quercetin in a dose-dependent manner, but no cell elongation was observed in wells treated with ellagic acid at the concentrations tested. Cell viability was tested qualitatively by placing a sample of the treated cells into a 96 well plate; cell growth was observed after incubation at 37°C for 48h. Cell viability was decreased by both ellagic acid and quercetin relative to the control.

Discussion

Cell Migration and Cytotoxicity. Cell migration was effectively inhibited by ellagic acid and quercetin, but was not significantly inhibited by anthocyanin, 80/20 fraction, or residue fraction. The inability of keracyanin to inhibit cell migration at the concentrations tested indicates that not all cyanidin glucosides possess equal anti-carcinogenic activity, or that cyanidin glucoside derivatives do not have the same level of activity as cyanidin glucosides themselves. Further research comparing keracyanin to cyanidin glucosides with known anti-carcinogenic activity will be necessary to determine the relationship. Neither of the BRB fractions (80/20 and residue), were effective migration inhibitors at the concentrations tested, so a synergistic effect was not observed. This could be because the active compounds in BRBs are not a large enough portion of the respective extracts to be effective at the tested concentrations, since ellagic acid and quercetin are both found in BRBs, and both effectively inhibited migration. Ellagic acid inhibited migration at lower concentrations than quercetin in both cell types, but there were significant differences in cytotoxicity between HUVECs and TE-8 cells. Ellagic acid was cytoxic for HUVECs at low concentrations, so would be the more effective of the two compounds at targeting HUVEC migration inhibition.

The observed differences in cytotoxicity and effective dose in each cell type indicate that ellagic acid and quercetin may act via different cellular mechanisms. Ellagic acid's lack of cytotoxicity in TE-8 cells suggests an altered molecular pathway may be present in this tumor cell line, which could allow this cell line to develop resistance against ellagic acid. This changed pathway may allow ellagic acid to inhibit cell migration at the same concentration in both cell types, while preventing cytotoxicity of the compound in TE-8 cells. Differences in dose-response were observed in the treatment

of HUVEC and TE-8 cells with quercetin. Equivalent inhibition of migration and cytotoxicity were achieved in TE-8 cells at twice the concentration used in HUVECs. This indicates that quercetin may be acting on the same molecular pathways in both cell types, but individual differences between cell lines exist. Quercetin's inhibition of migration and angiogenesis is consistent with previous research, which found quercetin acts upon COX-2 (cyclooxygenase-2) and NFκB[20]. Further experiments will be needed to test the molecular pathway hypothesis for both quercetin and ellagic acid. Gene expression of possible targets will be studied using RNA extraction, and RT-PCR.
Tubulogenesis. Since ellagic acid and quercetin inhibited HUVEC tubulogenesis at all concentrations tested, further study will be necessary to determine if a dose-response relationship exists. Inhibition of tubulogenesis was effective at equivalent or lower concentrations for both compounds examined, suggesting that targeting HUVEC angiogenesis may be more effective than targeting HUVEC migration. The cell elongation observed in the wells treated with quercetin, but not those treated with ellagic acid is consistent with the difference in effective dose between the two compounds in the migration assay. Further research will also be necessary to determine what step in the process of angiogenesis is inhibited. Analysis of gene expression during treatment with either ellagic acid or quercetin may indicate whether the anti-angiogenic effects of these compounds act via different mechanisms. Gene expression in the HUVEC tubulogenesis assay will also be compared to gene expression in the cell migration assay in order to determine if different pathways account for the differences in inhibitory concentration between the two assays. Further research may also include mixed cultures, in which HUVEC and TE-8 cells are combined in a spheroid to examine whether or not the tumor cells influence migration or tubulogenesis of HUVEC cells[21].

These results have shown that there are multiple compounds in black raspberries that can affect endothelial cell angiogenesis and cell migration. This provides the basis for future cell culture research on the interactions between endothelial cells in spheroids, and molecular research on the mechanisms involved in the inhibition of tubulogenesis and cell migration.

Figures

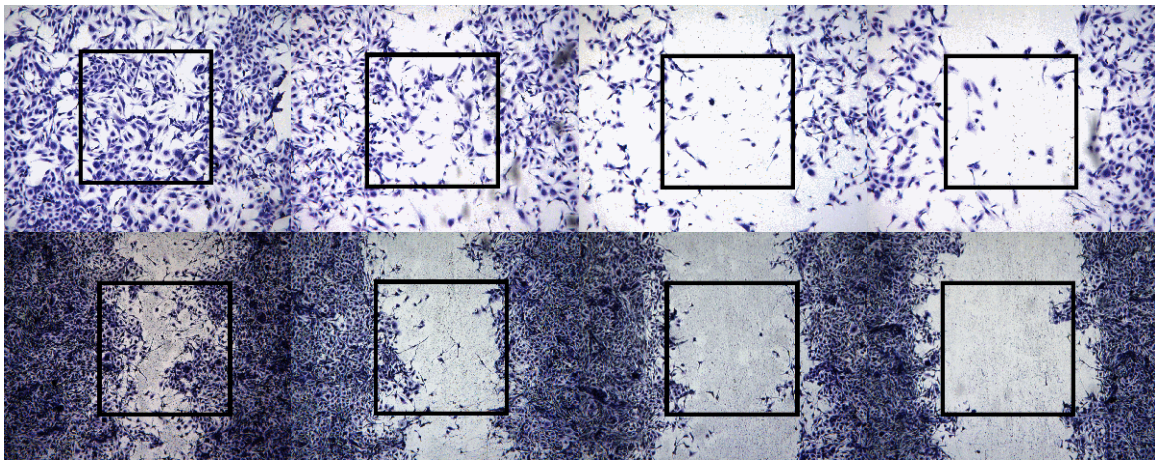

Figure 1. Cell migration of HUVECs (top row) and TE-8 cells (bottom row). The first column shows migration in excess of 100% of the control (top: anthocyanin at 20 µM, bottom: residue fraction at 1 µM). The second column shows the amount of migration observed in the controls. The third and fourth columns show migration at the lowest inhibitory concentration for ellagic acid (2 µM for both cell types) and quercetin (10 µM for HUVECs, 20 µM for TE-8 cells) respectively. The black boxes indicate an area 1x1mm in each image which was used to quantify cell migration relative to the control.

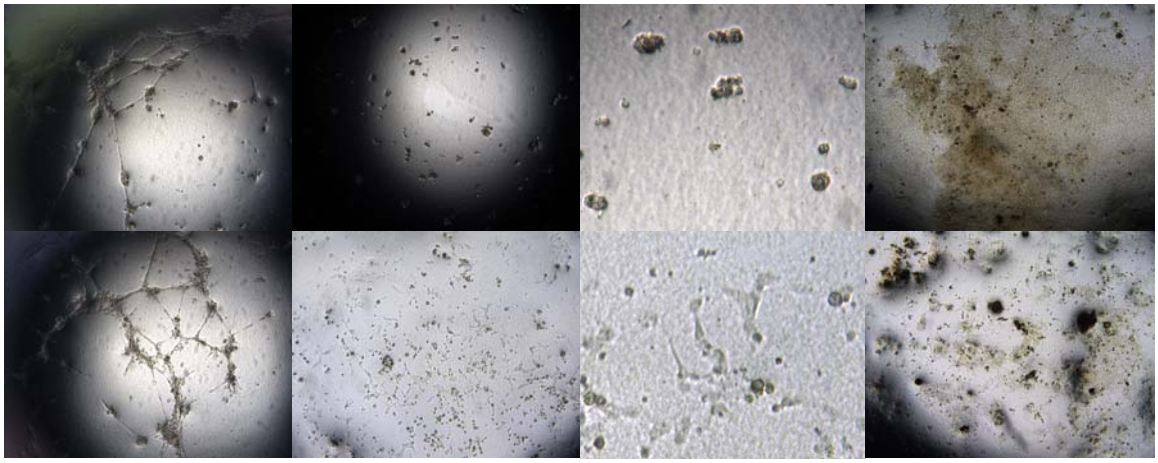

Figure 2. Tubulogenesis of HUVEC on a layer of Matrigel after treatment with ellagic acid (top) and quercetin (bottom). The left column shows extensive tube formation in the control wells. The second column from the left shows the complete inhibition of HUVEC tubulogenesis by both compounds at 2 µM. The third column from the left shows enlargements of the images at 2µM, indicating the different morphology of the HUVEC cells; ellagic acid inhibits cell elongation at 2 µM, while quercetin does not. The rightmost column shows the complete inhibition of HUVEC tubulogenesis at 100 µM for both compounds (some compound precipitation is evident in both images).

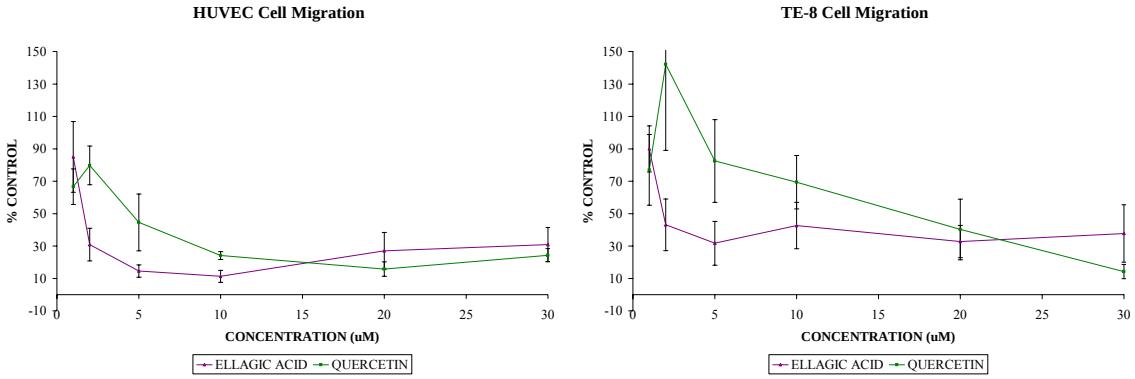

Figure 3. Migration of HUVEC and TE-8 cells as percent of control. The number of cells which migrated into a 1x1 mm area which had been scraped clean of cells after 24 hours was counted, and compared to the number of cells which migrated in the controls for each concentration. Human Umbilical Vascular Endothelial Cell (HUVEC) migration was inhibited by ellagic acid at 2 µM (30.9% control ± 10.1%, $P \leq 0.04$), and by quercetin at 10 µM (24.1% control ± 2.5%, $P \leq 0.02$). Migration of TE-8 cells was inhibited by ellagic acid at 2 µM (43.1% control ± 15.9%, $P \leq 0.04$), and by quercetin at 20 µM (40.3% control ± 18.7%, $P \leq 0.05$).

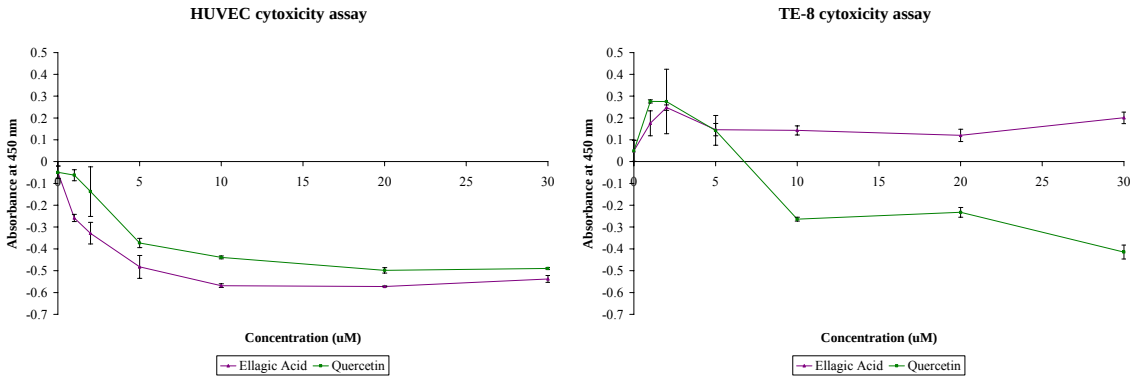

Figure 4. Graphs showing the absorbance at 450 nm after the CCK cytotoxicity assay. The left graph shows the dose-dependent cytoxic effects of ellagic acid and quercetin on HUVEC. The right graph shows the dose-dependent cytoxic effect of quercetin on TE-8 cells, and the resistance of TE-8 cells to ellagic acid. Ellagic acid showed cytotoxicity in HUVECs at 1 µM ($P \leq 0.008$), but was not cytoxic in TE-8 cells at the concentrations tested. Quercetin was cytoxic in HUVECs at 5 µM ($P \leq 0.002$), and was cytoxic in TE-8 cells at 10 µM ($P \leq 0.02$).

References

[1] Hecht, S., et al. 2006. Identification of cyanidin glycosides as constituents of freeze-dried black raspberries which inhibit *anti*-benzo[a]pyrene-7,8-diol-9,10-epoxide induced NFκB and AP-1 activity. *Carcinogenesis.* 27:1617-1626.

[2] Seeram, N., et al. 2006. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *J. Agric. Food Chem.* 54:9329-9339.

[3] Liu, Z., et al. 2005. Black raspberry extract and fractions contain angiogenesis inhibitors. *J. Agric. Food Chem.* 53:3909-3915.

[4] Hecht, S., et al. 2006. Identification of cyanidin glycosides as constituents of freeze-dried black raspberries which inhibit *anti*-benzo[a]pyrene-7,8-diol-9,10-epoxide induced NFκB and AP-1 activity. *Carcinogenesis.* 27:1617-1626.

[5] Liu, Z., et al. 2005. Black raspberry extract and fractions contain angiogenesis inhibitors. *J. Agric. Food Chem.* 53:3909-3915.

[6] Hecht, S., et al. 2006. Identification of cyanidin glycosides as constituents of freeze-dried black raspberries which inhibit *anti*-benzo[a]pyrene-7,8-diol-9,10-epoxide induced NFκB and AP-1 activity. *Carcinogenesis.* 27:1617-1626.

[7] Stoner, G., et al. 2006. Protection against esophageal cancer in rodents with lyophilized berries: potential mechanisms. *Nutrition and Cancer.* 54:33-36.

[8] Liu, Z., et al. 2005. Black raspberry extract and fractions contain angiogenesis inhibitors. *J. Agric. Food Chem.* 53:3909-3915.

[9] Hecht, S., et al. 2006. Identification of cyanidin glycosides as constituents of freeze-dried black raspberries which inhibit *anti*-benzo[a]pyrene-7,8-diol-9,10-epoxide induced NFκB and AP-1 activity. *Carcinogenesis.* 27:1617-1626.

[10] Labreque, L., et al. 2005. Combined inhibition of PDGF and VEGF receptors by ellagic acid, a dietary-derived phenolic compound. *Carcinogenesis.* 26:821-826.

[11] Tan, W-F., et al. 2003. Quercetin, a dietary-derived flavonoid, possesses antiangiogenic potential. *European J. of Pharmacology.* 459:255-262.

[12] Chen, T., et al. 2006. Black raspberries inhibit N-nitrosomethylbenzylamine (NMBA)-induced angiogenesis in rat esophagus parallel to the suppression of COX-2 and iNOS. *Carcinogenesis.* 27:2301-2307.

[13] Sagar, S., et al. 2006. Natural health products that inhibit angiogenesis: a potential source for investigational new agents to treat cancer—part 1. *Current Oncology.* 13:14-26.

[14] Dorai, T., and B. Agagrwal. 2004. Role of chemopreventive agents in cancer therapy. *Cancer Letters.* 215:129-140.

[15] Hanahan, D., and R. A. Weinberg. 200. The hallmarks of cancer. *Cell.* 100:57-70.

[16] Iiizumi, M., et al. 2007. Tumor-endothelial cell interactions: therapeutic potential. *Microvascular Research.* Doi:10.1016/j.mvr.2007.04.002.

[17] Iiizumi, M., et al. 2007. Tumor-endothelial cell interactions: therapeutic potential. *Microvascular Research.* Doi:10.1016/j.mvr.2007.04.002.

[18] Hecht, S., et al. 2006. Identification of cyanidin glycosides as constituents of freeze-dried black raspberries which inhibit *anti*-benzo[a]pyrene-7,8-diol-9,10-epoxide induced NFκB and AP-1 activity. *Carcinogenesis.* 27:1617-1626.

[19] Li, M., et al. 2002. Induction of retinoic acid receptor-β suppresses cyclooxygenase-2 expression in esophageal cancer cells. *Oncogene.* 21:411-418.

[20] Sagar, S., et al. 2006. Natural health products that inhibit angiogenesis: a potential source for investigational new agents to treat cancer—part 1. *Current Oncology.* 13:14-26.

[21] Korff, T., and H. Augustin. 1998. Integration of endothelial cells in multicellular spheroids prevents apoptosis and induces differentiation. *J. Cell Biology.* 143:1341-1352.